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Euphorbia escula L. Root and Root Bud Indole-3-Acetic Acid Levels at Three Phenologic Stages[1]

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ABSTRACT

Endogenous indoleacetic acid (IAA) levels of *Euphorbia esula* L. primary root and root buds were examined at three phenologic stages. High performance liquid chromatography coupled with fluorescence detection and gas chromatography-mass spectrometry, using $^{13}C_6$[benzene ring]-indole-3-acetic acid as internal standard, were used to measure root bud free and bound IAA levels in vegetative, full flower, and post-flower plants. Highest levels of free IAA (103 nanograms per gram fresh weight) were found in root buds during full flower. Esterified and amide IAA increased significantly in root buds of full flower and post-flower plants, but were not detectable in root buds of vegetative plants. Primary root-free IAA was highest in vegetative and full flower plants (34.5 nanograms per gram fresh weight) and decreased by 50% in post-flower plants.

Euphorbia esula L. is one of many plant species which has the capacity to produce adventitious shoots from root buds. Several plants with this capacity are horticulturally important, while others represent persistent and undesirable plants which interfere with the production of food and fiber. It was previously demonstrated that *E. esula* shows significant seasonal variation in the capacity of root buds to produce new shoot growth (18, 19). Root buds show the highest regenerative capacity in spring and fall with a significant reduction in regenerative capacity associated with full flower. A number of other plants, which reproduce vegetatively by root buds, show a similar reduction in the capacity of roots to produce new shoots during flowering (6, 7, 9–11, 17, 19, 22, 24).

It has been suggested that this reduction in shoot regenerative capacity during flowering is related to increased IAA levels in the root (9, 11, 23, 24). Early attempts to demonstrate a significant increase in root extractable IAA associated with flowering and active shoot growth in *Populus tremula* L. (9) and *P. tremuloides* L. (23) were successful. However, these studies did not utilize highly specific and sensitive techniques now available for IAA analysis, and root bud IAA was not measured. No attempt was made to examine conjugated IAA levels which may also fluctuate with phenologic stage.

The present study was undertaken to examine IAA levels of *E. esula* primary roots and adventitious root buds at three phenologic stages: vegetative, full flower, and post-flower. Since the capacity of *E. esula* root buds to produce new shoots appears related to the plants' phenologic stage (18, 19), we hypothesized that the levels of IAA in roots and/or root buds might also show significant changes between these stages.

MATERIALS AND METHODS

Instrumentation. Quantitation of IAA was done by HPLC coupled with fluorescence detection. The HPLC was a Kratos 430 gradient former, 400 pump, 970 fluorometer, and Shimadzu R-C3A computing integrator. Excitation was at 220 nm and emitted light was monitored using a 350 nm bandpass filter before the photomultipler tube. Results of the HPLC fluorescence procedure were verified by analyzing several samples by GC-MS using $^{13}C_6$-[benzene ring]-indole-3-acetic acid (gift from Dr. Jerry Cohen, USDA, Beltsville, MD) as internal standard. The mass spectrometer was a 70 eV VG 7070E-HF coupled to a Varian 3700 GC. The GC was equipped with a 30 m × 0.025 mm DB-5 (J and W) capillary column with a helium velocity of 30 cm/s and temperature programmed to hold at 50°C for 4 min and increased to 220°C at 5°C/min. Splitless mode injections were made. The mass range scanned was 100 to 250 m/z at 1/2 s/decade.

Plant Material. *Euphorbia esula* L. plants were cloned by root cuttings from a single plant and propagated as previously described (18). After 3 months plantlets were transferred to polyvinyl chloride tubes 0.1 m diameter and 1 m long filled with 50:50 (v/v) mixture of potting soil and sand. Plants used in these experiments were at least 1 year old with root systems extending the entire length of the tube. Plants were grown under greenhouse conditions, watered daily, and fertilized once each week with commercial liquid fertilizer (Pete's Professional, 20-20-20). Supplemental lighting was provided during winter months.

Experimental Design. Free and conjugated IAA levels were determined for root bud tissue of *E. esula* at three phenological stages: vegetative, full flower, and post-flower. Primary root free IAA was also measured. At each growth stage root buds and primary root tissue were collected from three to four plants and analyses were run in triplicate. Experiments were repeated with similar results. Tissue samples were collected at approximately the same time each day. Root bud sample sizes ranged from 200 to 300 mg, FW,[3] while primary root samples ranged from 2.7 to 3.0 g FW.

Quantitation of IAA. The same procedure was used for isolation of IAA for both HPLC and GC-MS. Freshly harvested samples were weighed and frozen immediately in liquid N_2. Frozen tissue was ground to a fine powder in a mortar and pestle. A 2 ml volume of 80% methanol/water (v/v) was added along with 11,000 dpm of methylene [^{14}C]IAA (Amersham, 59 mCi/mmol) which was equivalent to 15 ng of IAA. For samples to be

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[3] Abbreviation: FW, fresh weight; BHT, butylated hydroxytoluene; SPE, solid phase extraction.

analyzed by GC-MS, 632 ng of [$^{13}C_6$]IAA were added instead of radioactive IAA. The tissue was homogenized for 1 min before being quantitatively transferred to test tubes with an additional 13 ml of 80% methanol. Tissue samples were extracted overnight in the dark at 4°C.

Samples were evaporated to the aqueous phase under a stream of nitrogen at 40°C. The procedure of Bandurski and Schulze (2) was used to hydrolyze esterified and amide IAA. The aqueous phase, containing free or free plus conjugated IAA, was transferred to 250 ml Erlenmeyer flasks by rinsing the test tubes with 100 ml of 0.075 M K_2HPO_4. The pH was lowered to 2.7 with 8.4 N H_3PO_4. The procedure of Liu and Tillberg (16) was then used to partition IAA using dialysis. The only modifications made in this procedure were volume reductions of the three phases. Phase I extraction buffer (0.075 M K_2HPO_4, pH 2.7) was reduced to 100 ml, phase II (diethyl ether plus BHT) was reduced to 130 ml, and phase III dialysis tubing buffer (0.1 M K_2HPO_4, pH 9.0) was reduced to 15 ml. Samples were dialyzed in the dark for 12 to 15 h at room temperature.

After partitioning, phase III dialysis tubing buffer was transferred to 20 ml test tubes and acidified to pH 2.7 with 8.4 N H_3PO_4. IAA was extracted from the acidified dialysis tubing buffer using 6 ml high capacity C_{18} SPE columns (JT Baker, Phillipsburg, NJ). SPE columns were first conditioned with 6 ml of HPLC grade methanol and followed by 6 ml of 0.1 M K_2HPO_4 (pH 2.7). An additional 6 ml of buffer was added with the vacuum turned off. Sample reservoirs (15 ml) were attached and acidified dialysis tubing buffer added. Samples were aspirated through columns over a period of 5 min. Columns were washed with 2 ml of 20% methanol/water (v/v) (pH 2.7) and air dried with vacuum for 3 min. IAA was eluted with two-1 ml aliquots of 80% methanol/water (v/v). The IAA fraction was collected in 2 ml conical centrifuge tubes and evaporated to dryness under a stream of N_2 at 40°C. Samples were resuspended in 100 μl of HPLC grade methanol for HPLC quantitation or 20 μl for purification by HPLC and quantitation by GC-MS.

HPLC solvents used were acetonitrile (American Burdick and Jackson, Muskegon, MI) and water (EM Science, Cherry Hill, NJ) containing 0.1% (v/v) glacial acetic acid (JT Baker) (pH 3.3). The column was a 250 by 4.6 mm 5 μm Bakerbond C_{18} held at 30°C by a recirculation water bath. The gradient former was programmed to hold for 1 min at 95/5 water/acetonitrile (v/v), followed by a 5 min linear gradient to 80/20 water/acetonitrile (v/v) and hold for 20 min. At a flow rate of 1.5 ml/min IAA had a retention volume of 22.5 ml and K′ of 6.9. Plate number/meter calculated for IAA under conditions described was 45,000. The injection volume was 10 or 20 μl depending on IAA concentration. IAA quantitation was by comparison of peak area to standard curves of authentic IAA and corrected for losses by isotope dilution (20). Actual samples were also spiked with known amounts of IAA to check the validity for the standard curve. The limit of detection under our conditions was 0.25 ng/10 μl injection, however this was not the limit of instrument sensitivity.

Samples quantified by GC-MS were purified by HPLC using the same conditions described above. The entire 20 μl sample was injected. IAA peaks were collected with the fluorometer excitation beam momentarily interrupted by a shutter to avoid any photodecomposition. One ml C_{18} SPE columns were used to extract IAA from the 80% aqueous HPLC solvent by first diluting the IAA fraction with 5 ml of HPLC grade water acidified to pH 2.7 with 8.4 N H_3PO_4. This step was necessary to reduce the concentration of acetonitrile below 5% (v/v). One ml columns were first conditioned with 2 ml of HPLC grade methanol, followed by 2 ml of 96/3 water/acetonitrile (v/v) (pH 2.7). Vacuum was turned off and 1 ml of 96/3 water/acetonitrile (v/v) was added. Sample reservoirs were attached. IAA fractions were added to reservoirs and aspirated through the columns at a flow rate of 2 to 3 ml/min. Since no buffer salts were present, columns were not washed but were allowed to air dry under vacuum. IAA was eluted with two 100 μl volumes of HPLC grade methanol collected in 2 ml conical centrifuge tubes. Diethyl ether was added to the centrifuge tubes and IAA was methylated with diazomethane.

Quantitation by GC-MS was done by comparison of the ratio of mass 130/136 (base peak of endogenous IAA and [$^{13}C_6$]IAA internal standard) and verification of the analysis by the ratio of 189/195 (molecular ion of IAA-methyl ester and [$^{13}C_6$]IAA methyl ester) as suggested by Cohen *et al.* (5). Comparing the authentic IAA mass spectra to the internal standard indicated that only about 90% of the added internal standard existed as the uniformally labeled [$^{13}C_6$]IAA. Calculation of endogenous IAA reflect this difference. IAA levels determined by GC-MS do not reflect contributions from naturally occurring stable isotopes of carbon and nitrogen. This probably has resulted in a slight under estimation of endogenous levels.

RESULTS

Typical HPLC chromatograms of primary root and root bud-free IAA extracts are shown in Figure 1. IAA eluted without interfering peaks and samples spiked with known amounts of authentic IAA increased in area proportional to the amount of IAA added. GC-MS verified the presence of IAA in the HPLC peak and IAA levels determined by GC-MS showed good agreement with those analyzed by HPLC. For example, post-flower root tissue-free IAA levels were determined to be 13.6 ng/g FW by GS/MS and 15.3 ng/g FW by HPLC (Table I). Because of the limited availability of root buds, verification by GC-MS was most often performed on root tissue samples. Ester and amide conjugates levels were verified at random by single GC-MS samples (data not shown), while triplicate GC-MS samples of root and root bud free IAA were included in each sample run (Table I). Figure 2 shows a typical mass spectrum from which free IAA of a post-flower root tissue sample was calculated. The ratio of 130/136 is 0.072 and the ratio of 189/195 is 0.072 verifying the analysis (5). Based on the amount of internal standard added (632 ng × 0.9 = 568 ng) and the sample weight (3 g) the endogenous IAA content is calculated to be 13.6 ng/g FW. Although an additional derivation step is required, $^{13}C_6$[benzene ring]-indole-3-acetic acid worked very well for purposes of verification of the fluorescence assay, as suggested by Cohen *et al.* (5).

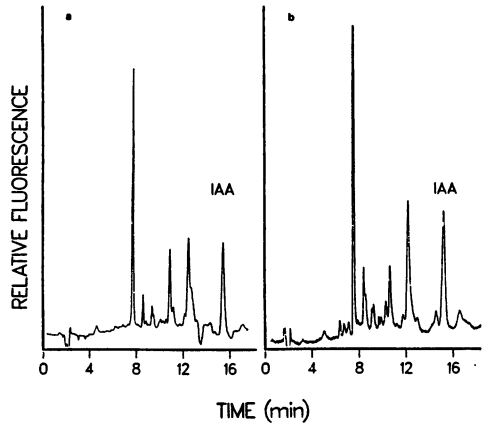

FIG. 1. Typical HPLC/Fluorescences chromatograms of *E. esula* tissue extracts analyzed for indole-3-acetic acid; (a) root bud tissue, (b) primary root tissue. The IAA peak was identified by cochromatography with authentic-IAA, spiking tissue samples and GC-MS.

Table I. *Levels of Free and Bound Forms of Indole-3-acetic Acid in Primary Root and Root Bud Tissue of E. esula L. at Three Different Phenological Stages*

Tissue and IAA Form	Phenological Stage	IAA[a]
		ng/g fresh wt
Root bud-free IAA		
	Vegetative	74.8 (7.4)
	Full flower	103.8 (10.9)
		94.9 (2.5)[b]
	Postflower	80.5 (10.1)
Root bud esterified IAA		
	Vegetative	ND[c]
	Full flower	15.5 (0.4)
	Postflower	44.5 (13.3)
Root bud amide IAA		
	Vegetative	ND
	Full flower	66.5 (8.0)
	Postflower	136.0 (6.7)
Primary root-free IAA		
	Vegetative	35.0 (1.3)
	Full flower	34.3 (0.1)
	Postflower	15.3 (0.2)
		13.6 (1.7)[b]

[a] Values listed are the means (SE) of triplicate samples. [b] Levels of IAA determined by GC-MS. [c] None detected, *i.e.* the levels of IAA determined after treatment with 1 N and 7 N NaOH were not significantly different than the amount determined for free IAA.

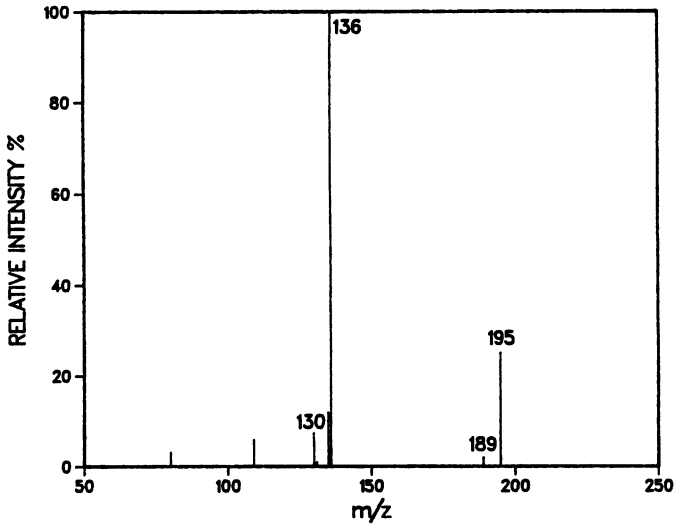

FIG. 2. Typical mass spectra of post-flowering root tissue sample spike with [$^{13}C_6$]IAA as internal standard. The base peak and molecular ion of endogenous IAA-methyl ester and [$^{13}C_6$]IAA methyl ester are shown. The ratio of 130/136 and 189/195 is 0.072. Sample weight 3 g and 632 ng of internal standard was added.

Free IAA levels were significantly higher in root buds of *E. esula* plants in full flower (Table I) as compared to vegetative and post-flower plants. IAA conjugates were undetectable in root buds of vegetative plants, while esterified and amide IAA was found in root buds of full flower and post-flower plants. Highest levels of conjugated IAA were found in post-flower root buds. The ratio of esterified to amide IAA resembled that of legumes in which amide conjugates are most abundant (2). Free IAA levels in root tissue were highest in vegetative and full flower plants and decreased by 50% in post-flower plants.

DISCUSSION

There is little available information concerning free and conjugated IAA levels in root buds of perennial plants. Hillman *et al.* (14) determined the IAA content of *Phaseolus vulgare* L. axillary buds from intact and decapitated plants. Free IAA levels in these axillary buds were reported to range from 60 to 130 ng/g dry weight. Expressed on a dry weight basis, *Euphorbia esula* root bud-free IAA levels would be 3 to 4 times higher (average water content of root buds is 77%). *Chrysanthemum morifolium* shoot tip IAA levels ranged from 15.6 to 29.0 ng/g FW, which is also considerably lower than *E. esula* root bud-free IAA levels. On the other hand, *E. esula* primary root-free IAA levels were very similar to levels of free IAA reported for primary root tissue of *Zea mays* (21), vegetative tissue of *Z. mays*, *Pisum sativum* (1, 2), and *Avena sativa* (2), and also similar to free IAA levels in stem cuttings of *C. morifolium* (25).

The results of the present study suggest that the reduced elongation of *E. esula* root buds during flowering (18, 19) was associated with significant increases in root bud-free IAA levels. The possible existence of this relationship has been theorized (11, 17), and this theory supported by evidence from studies utilizing *Avena* bioassays to quantify IAA levels in roots to *Populus tremula* (9) and *P. tremuloides* (22). Our work represents the only attempt to examine the relationship of plant phenology to root bud endogenous IAA levels using highly specific and sensitive techniques like HPLC/fluorescence and GC-MS. Harrison and Kaufman (13) attempted to demonstrate a relationship between free and conjugated IAA and zeatin levels and tiller bud release in *Avena sativa* associated with inflorescence emergence. No relationship to IAA levels was found. However, since stem segments which included a tiller bud were analyzed, rather than tiller buds directly, it is possible that differences in IAA levels occurred but were masked by the large amount of vegetative tissue included in the sample. *E. esula* root tissue-free IAA levels were found to be considerably lower than that of root buds, and had large amounts of vegetative tissue been included in estimates of root bud IAA, changes in IAA levels could have been masked.

Levels of IAA conjugates did not appear to be related to phenologic variation in *E. esula* root bud growth. Ester and amide IAA levels increased dramatically in full flower and post-flower root buds. Root buds of post-flower plants had the highest levels of conjugated IAA; however, root buds excised from these plants elongate rapidly (18). This seems to indicate that conjugated IAA is not directly related to shoot production by root buds. It has been suggested that IAA conjugates have four possible functions: (a) storage, (b) transport, (c) protection from enzymic hydrolysis, and (d) regulation of free IAA levels (4, 12). The function of IAA conjugates in root buds of *E. esula* was not evident from our experiments.

Primary root-free IAA levels appeared to reflect the growing state of the main shoot but did not appear directly related to adventitious shoot production. Highest free IAA levels were associated with very active stem growth of vegetative and full flower plants. Post-flower plant stems show considerable axillary bud growth indicating reduced IAA translocation from the shoot apex. The 50% reduction in primary root-free IAA levels would appear to reflect this decrease in auxin production and the overall reduction in plant vigor which occurs at this phenologic stage.

Little information is available on the endogenous IAA levels in root buds and their possible relationship to root bud growth. However, research utilizing exogenous IAA provides evidence implicating IAA as a factor in control of adventitious shoot production by root buds. Exogenous IAA at concentration greater than 10 μM significantly reduced root bud elongation in *P. tremula* (8), *Chondrilla juneca* L. (15), *Chameanerion angustifolium* L. (11), and *E. esula* (3, 18). In addition, auxin transport inhibitor studies indicate that IAA moving basipetally from the shoot is involved (9, 18). In *Rubus idaeus* L., root cuttings will not produce adventitious shoots when plants are flowering. Marston and Village (17) demonstrated that if the shoot apex and

axillary buds were removed 3 weeks before root cuttings were taken, adventitious shoot production was significantly increased. This treatment's effectiveness may result from reducing the amount of IAA translocating from shoot to root.

The suggestion was previously made that *E. esula* root buds might be an excellent source of material for studies of apical dominance using exogenous plant growth regulators (3). The present work further indicates that they would also provide an excellent system for the study of endogenous IAA regulation in meristemic tissue.

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